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PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
C12N 15/12, C07K 14/495, A61K 38/18	A1	WO 97/06254
		(43) International Publicati n Date: 20 February 1997 (20.02.97)

(21) International Application Number: PCT/EP96/03427

(22) International Filing Date: 2 August 1996 (02.08.96)

(30) Priority Data:
95112241.5 3 August 1995 (03.08.95) EP
(34) Countries for which the regional or international application was filed: DE et al.

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description.
Date of receipt by the International Bureau:
16 September 1996 (16.09.96)

(54) Title: HUMAN MP52 ARG PROTEIN

(57) Abstract

This invention relates to a human MP52 Arg and a pharmaceutical medical composition inter alia for promoting cartilage and bone morphogenation comprising human MP52 Arg. In particular, the medical composition is useful for treating bone diseases caused by abnormal bone metabolism such as osteoporosis, for treating bone fracture and for the purpose of orthopedic reconstruction, bone transplantation, cosmetic surgery and dental therapeutics. Further, it is useful for treating cartilage disorders.

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HUMAN MP52 ARG PROTEIN

This invention relates to a new compound human MP52 Arg and a pharmaceutical medical composition inter alia for promoting cartilage and bone morphogenation comprising human MP52 Arg. In particular, the medical composition is useful for treating bone diseases caused by abnormal bone metabolism such as osteoporosis, for treating bone fracture and for the purpose of orthopedic reconstruction, bone transplantation, cosmetic surgery and dental therapeutics. Further, it is useful for treating cartilage disorders.

Pharmaceutical compositions including vitamin D3, calcitonin, estrogen and bisphosphonate derivatives have been used in clinical practice for treating bone diseases. Their therapeutic results, however, are not entirely satisfactory, and a better pharmaceutical composition is highly desired.

The TGF-ß family of growth factors comprising BMP, TGF, and inhibin related proteins have been reported to be useful for wound healing and tissue repair. The bone morphogenetic activity of some of those proteins has been also known. PCT patent application WO 93/16099 and WO 95/04819 disclose DNA sequences encoding human TGF-ß-like proteins, and as a preferred protein human MP52.

E. E. Storm et al. reported in Nature, 1994, vol.368, p.639-643 that mutations of mouse growth/differentiation factor 5 (GDF5) gene, a new member of the TGF-ß superfamily, cause mice brachypodism. Mouse GDF5 has the same amino acid sequence of the predicted mature form as that of human MP52 except one amino acid. However, there is no suggestion in this publication to use those proteins for the treatment of bone diseases.

It was therefore the object of the present invention to provide a further growth factor that is useful as an agent for the stimulation of bone or cartilage formation.

One embodiment of the present invention is therefore protein human MP52 Arg comprising amino acids 1 to 121 of SEQ ID NO: 1.

In fact, it has surprisingly been found by the invention that there are cell lines which, when expressing a suitable DNA sequence, form human MP52 Arg or a mixture of human MP52 and human MP52 Arg. For the first time the invention succeeded in providing human MP52 Arg which has bone morphogenetic activity and is useful for preventing and/or treating bone diseases.

It has also been confirmed that human MP52 Arg induces formation of cartilage from undifferentiated mesenchymal cell and stimulates the differentiation and maturation of osteoblasts. Therefore, human MP52 Arg is effective for preventing and/or treating bone diseases caused by abnormal bone metabolism such as osteoporosis. It also accelerates the healing process of bone fractures. Moreover, it is useful for orthopedic reconstructions, bone transplantations and dental therapeutics because of its bone morphogenetic activity. Furthermore, MP52 Arg is effective for preventing and/or treating cartilage disorders caused by abnormal cartilage metabolism.

A further object of the present invention is a process for the production of human MP52 Arg, wherein at least a part of a DNA sequence as shown in Sequence ID NO:1 is introduced into a suitable host cell under conditions favouring expression of the DNA sequence and protein formation, followed by isolation of said protein from other proteins produced by said host cell.

Within the framework of the present invention the DNA sequence depicted in SEQ ID NO:1 may be used for producing human MP52 Arg, however also shorter portions thereof, provided they still encode human MP52 Arg and expression of the DNA sequence in a suitable vector/host cell system is possible. Suitable expression systems are known to a person skilled in the art and it can easily be determined by routine experimentation what the minimum

requirements for the length of the DNA sequence of SEQ ID NO:1 are.

Subsequent to protein formation the proteins are recovered from the host cell by methods known per se and finally human MP52 Arg is isolated therefrom. In particular the isolation of human MP52 and MP52 Arg, which differ from one another only in respect of one amino acid, can be carried out using very precisely differentiating separation methods which are known to a person skilled in the art. One example is the electrophoretic separation of MP52 Arg performed according to the method of Davis (Ann. NY Acad. Sci., 121, 404-427, 1964) with small modifications lik addition of 0.1 % Nonidet P-40 and 6 M urea. After electrophoresis the separated MP52 Arg is electroeluted from th gel pieces in the same buffer.

A further object of the present invention is a pharmaceutical composition containing human MP52 Arg. Optionally, this composition may also include usual carrier substances, auxiliary substances, diluents and/or fillers. The pharmaceutical composition according to the invention is useful for promoting bone morphogenation, treatment or prevention of damage to bone, cartilage, connective tissues, skin, mucous membranes, epithelium or teeth, for application in dental implants and for application in wound-healing and tissue regeneration processes.

For the treatment of bone diseases caused by abnormal bone metabolism, human MP52 Arg is administered in systemic by injection such as intravenous injection, muscle injection and intraperitoneal injection, oral administration, non-oral administration such as suppository, and other any conventional methods.

For the treatment of bone fracture, it is administered in systemic and locally by injection, oral and non-oral administration. Also matrix containing human MP52 Arg is preferably implanted in the area close to the fractured bone.

Suitable matrixes are natural polymers such as collagen and fibrin clot, and artificial polymers degradable in living body such as polylactated glycolic acid.

In case of orthopedic reconstruction, cosmetic surgery, bone transplantation and dental implantation, human MP52 Arg for example can be coated on the surface of bone and tooth to be implanted by means of collagen paste, fibrin glue and other adhering materials. It can also be applied to the tissue, the bone or alveolar bone around which the bone and tooth is transplanted. In case of bone transplantation it can be used for both natural and artificial bone. As the material of artificial bone and tooth, conventional materials such as metals, ceramics, glass and other natural or artificial inorganic substance are used. Hydroxyapatite is a preferred artificial substance. Artificial bone can be constructed by dense material in the inner part and porous material in the other part. For example, d nse steel covered porous steal can be cited. Porous hyroxyapatite is one of the materials to produce artificial bone. When such porous material is used, human MP52 Arg can be penetrated into it. The surface of artificial bone can also be roughened to keep human MP52 Arg on the surface.

It is also beneficial to apply human MP52 Arg to the part from which cancerous bone tissue is removed in order to accelerate th reconstruction of bone.

The dose of human MP52 Arg to be obtained is decided depending upon the purpose and the method of application. In general, when it is administered in systemic, the dose is from 1 μg to 100 μg/kg. When it is used for implantation, the preferred dose is from 30 μg to 30 mg per site.

This purified human MP52 Arg can be formulated in any conventional form such as injection liquid, pills, capsules and suppository. For local administration human MP52 Arg can b included in a matrix such as collagen, fibrin glue and poly

lactated glycolic acid. For the use of implantation and transplantation it is applied to the surface or in the porous part of bone and tooth.

This invention is illustrated by the examples.

Example 1

Construction of expression vector for MP52 Arg

The pSK52s plasmid (WO 95/04819) was digested with Hind III and the DNA fragment containing the cDNA comprising the complete coding region for MP52 was isolated by extraction from 0.8 % low melting agarose gels and ligated into the Hind III site of pABstop vector, which is supplied by Dr. Gerd Zettlmeißl of Behringwerke AG. The structure of the resulting MP52 expression vector, pMSS99 (5.0 kb), was confirmed by the DNA sequencing and the restriction enzyme mapping. The genetic elements of pMSS99 are schematically shown in Fig.1. The MP52 sequence in pMSS99 comprises the nucleotides 576-2279 in SEQ ID NO:1 of the Sequence Listing.

Example 2

Establishment of CHO clones producing MP52 Arg

Dhfr-deficient CHO cells CHO-DUKX-B11 (Urlaub, G. and Chasin, L.A. (1980) Proc.Natl.Acad.Sci, USA, 77, pp.4216-4220) were cotransfected with the expression plasmid for MP52 (pMSS99) and pSVOAdhfr (Zettlmeißl, G. et al., (1987) Bio/Technology 5, 720-725) by calcium phosphate-mediated DNA transfer method. Then high producer clones of MP52 Arg were established by a gene amplification protocol using methotrexate (MTX).

Briefly, 10 μg of pMSS99 and 2 μg of pSVOAdhfr were dissolved in

1 ml of 25 mM HEPES-140 mM NaCl-0.75 mM Na_2HPO_4 (pH 7.05), then mixed with 50 µl of 2.5 M $CaCl_2$. The resultant precipitates were overlaid to CHO-DUKX-B11 cells and incubated at room temperature for 30 min. Then MEM ALPHA medium with ribo- and deoxyribo-nucleosides (MEMα⁺) containing 10 % fetal bovine serum (FBS) was added to the cell layer to incubate in CO_2 incubator for 4-6 h. After a treatment with 10 % glycerol in MEMα⁺ containing 10 % FBS at room temperature for 3 min, the cells were cultured in MEMα⁺ containing 10 % FBS for 2 days. Then the cells were placed in MEM ALPHA medium without ribo- and deoxyribo-nucleosides (MEMα⁻) containing 10 % dialyzed FBS to select the transformants. The transformant clones were isolated and assayed for the expression of MP52 Arg by Western blot analysis as described in the next section.

Then high producer clones of MP52 Arg were established by a gen amplification protocol using methotrexate (MTX). The MP52 Arg producing clones were further selected stepwisely in increasing concentrations of methotrexate (MTX) to amplify the MP52 gene in accordance with the pSVOAdhfr gene. Several clones were obtained which produced 1-3 µg of mature MP52 Arg/10^6 cells/24 h at 400 nM MTX.

Example 3

Detection of MP52 Arg in the culture supernatants

Clones were examined for the expression of MP52 Arg by Western blot analysis as follows: The culture supernatants (1-15 µl) were applied on SDS-PAGE (15-25 % polyacrylamide gradient gel, Daiichi Pure Chemicals) under reducing conditions, then the proteins were transferred to a PVDF membrane (Clear Blot Membrane-P, ATTO). The membrane was blocked with Block Ace (Dai-Nihon Seiyaku) for 1 h, rinsed with Tris-buffered saline (TBS), then treated with 10 µg/ml of chicken antibodies to MP52 Arg in 10-times diluted Block Ace overnight. After washing the membrane with 0,1 % Tween 20 in

TBS (TTBS), the membrane was treated with rabbit anti-chicken IgG-ALP conjugate (Sigma A 9171) in 10-times diluted Block Ace for 1 h. The membrane was washed with TTBS and then reacted with Alkaline phosphatase Conjugate Substrate Kit (BIO-RAD) to visualize the bands corresponding to MP52 Arg.

Example 4

Cell culture of the MP52 Arg-producing CHO cell line

The CHO cell line with the highest productivity of MP52 Arg and MP52, MC-2 (deposited under No.FERM BP-5142 on June 21, 1995 with the National Institute of Bioscience and Human Technology, Japan) was grown with roller bottles containing MEMα⁻ supplemented with 10 % FBS, 400 nM MTX, 100 U/ml Penicillin and 100 µg/ml Streptomycin. After the MC-2 cells had grown to confluency, they were washed with serum-free MEMα-n and then cultured in serum-free DME/F12 medium supplemented with 10 mM HEPES (pH 7.3), 10 KIE/ml Aprotinin, 1 mM sodium butyrate, 6 µg/ml sodium selenate, 5 µg/ml transferrin, 18 µg/ml ethanol amine, 9 µg/ml insulin, 100 U/ml Penicillin and 100 µg/ml Streptomycin. Th conditioned medium was collected every day for a week.

Example 5

Purification

One liter of the culture supernatants were mixed with 0.1 volume of 0.2 M sodium phosphate buffer, pH 6.0, and applied to a POROS HS column (10 ml, PerSeptive Biosystems). The elution was performed by a linear gradient of NaCl from 0.3 to 2 M. The eluate containing MP52 Arg was applied to reverse-phase column (RESOURCE RPC, Pharmacia). The elution was performed by linear gradient of acetonitrile, containing 0.05 % TFA and MP52 Arg was eluted at about 35 % acetonitrile. The N-terminal amino acid

sequence analysis for purified MP52 Arg was performed using a pulse liquid gas phase sequencer (Applied Biosystems model 476). The result is shown in Table 1. It is indicated that MP52 Arg is processed proteolytically at Arg(380)-Arg(381) (amino acid positions -1 and +1 of Sequence ID NO:1) from its precursor. However, the precursor can also be processed at Arg(381)-Ala(382) (amino acid positions +1 and +2 of Sequence ID NO:1).

Table 1

N-Terminal Amino Acid Sequence Analysis of MP52 Expressed in CHO-Cells

Cycle	Amino Acid (pmol)	Amino Acid Sequence of MP52
#1	Arg(32.7),Ala(37.2)	Arg
#2	Ala(41.1),Pro(19.0)	Ala
#3	Pro(30.7),Leu(24.2)	Pro
#4	Leu(34.6),Ala(19.8)	Leu
#5	Ala(34.0),Thr(9.1)	Ala
#6	Thr(12.3),Arg(13.7)	Thr
#7	Arg(24.5),Gln(10.1)	Arg
#8	Gln(17.8),Gly(15.4)	Gln
#9	Gly(24.7),Lys(14.8)	Gly
#10	Lys(25.9),Arg(16.1)	Lys
#11	Arg(23.2),Pro(13.1)	Arg
#12	Pro(20.0),Ser(3.2)	Pro
#13	Ser(4.9),Lys(11.2)	Ser
#14	Lys(16.6),Asn(7.2)	Lys
#15	Asn(12.1),Leu(8.7)	Asn
#16	Leu(18.3),Lys(14.7)	Leu
#17	Lys(20.9),Ala(10.8)	Lys
#18	Ala(15.5),Arg(10.9)	Ala
#19	Arg(15.4)	Arg
#20	Ser(2.6)	Cys
#21	Ser(3.26),Arg(10.8)	Ser
#22	Arg(18.4),Lys(7.0)	Arg
#23	Lys(10.8),Ala(7.2)	Lys
#24	Ala(13.0),Leu(10.3)	Ala
#25	Leu(14.5),His(3.2)	Leu
#26	His(6.8),Val(9.1)	His
#27	Val(14.2),Asn(8.0)	Val
#28	Asn(8.7),Phe(9.5)	Asn
#29	Phe(9.9),Lys(8.3)	Phe
#30	Lys(1.0)	Lys

Example 6

Biological activity

Osteoprogenitor-like ROB-C26 cells (Calcif. Tissue Int. vol. 49, p. 221-225, 1991) were plated onto 48-well multi-well plates (Coaster) at a density of 1.5×10^4 cells/well and pre-incubated for 3 days in MEMα⁻ containing 10% fetal bovine serum (FBS). After the removal of culture medium, fresh MEMα⁻ containing 10% FBS and serially diluted MP52 Arg in 10mM HCl (2µl/ml) was added to the cultures and incubated for 6 days with changing the medium and the additives on day 3. Cell layers were washed with phosphate-buffered saline, and extracted with 0.2% Nonidet containing 1 mM $MgCl_2$. Alkaline phosphatase (ALP) activities were determined according to the procedure of Takuwa et al. (Am. J. Physiol. vol. 257, p. E797-E803, 1989). As shown in Table 2, the treatment of ROB-C26 cells with MP52 Arg increased total ALP activities per well concentration-dependently.

Table 2

Influence of CHO cell-derived MP52 Arg on the ALP activity of the ROB-C26 cell line

Concentration of MP52 Arg (ng/ml)	ALP activity (nmol/min/well)
Vehicle (10 mM HCl) - treated control	5.22 ± 0.19
3	5.79 ± 0.62
10	7.10 ± 0.75*
30	8.36 ± 0.77*
100	9.35 ± 0.67*
300	10.34 ± 1.06*
1000	16.09 ± 1.26*

Values represent means ± S.D. of 4 cultures. *$p<0.01$ compared to the vehicle-treated control (Dunnett's test).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Biopharm Gesellschaft zur biotechnologischen Entwicklung von Pharmaka mhH
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(F) POSTAL CODE (ZIP): 69115

(ii) TITLE OF INVENTION: New Protein Human MP52 Arg

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 95112241.5

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2703 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:640..2142

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION:640..720

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:1780..2142

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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TTCCTGAGTT CAGGTTTGTA AAAGATTTTT CTGAGCACCT GCAGGCCTGT GAGTGTGTGT 180

GTGTGTGTGT GTGTGTGTGT GTGTGTGTGA AGTATTTTCA CTGGAAAGGA TTCAAAACTA 240

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CGGTTGGCTT TCTCCTTTCA AGAACGAGTT ATTTTCAGCT GCTGACTGGA GACGGTGCAC      420

GTCTGGATAC GAGAGCATTT CCACTATGGG ACTGGATACA AACACACACC CGGCAGACTT      480

CAAGAGTCTC AGACTGAGGA GAAAGCCTTT CCTTCTGCTG CTACTGCTGC TGCCGCTGCT      540

TTTGAAAGTC CACTCCTTTC ATGGTTTTTC CTGCCAAACC AGAGGCACCT TTGCTGCTGC      600

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                                            Met Arg Leu Pro Lys
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                -115                -110                -105

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-55                 -50                 -45                 -40

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            -20                 -15                 -10

TTC AGC CAG CGG CGA AAA CGG CGG GCC CCA CTG GCC ACT CGC CAG GGC      1806
Phe Ser Gln Arg Arg Lys Arg Arg Ala Pro Leu Ala Thr Arg Gln Gly
        -5                  1                   5

AAG CGA CCC AGC AAG AAC CTT AAG GCT CGC TGC AGT CGG AAG GCA CTG      1854
Lys Arg Pro Ser Lys Asn Leu Lys Ala Arg Cys Ser Arg Lys Ala Leu
 10                  15                  20                  25

CAT GTC AAC TTC AAG GAC ATG GGC TGG GAC GAC TGG ATC ATC GCA CCC      1902
His Val Asn Phe Lys Asp Met Gly Trp Asp Asp Trp Ile Ile Ala Pro
                30                  35                  40
```

CTT GAG TAC GAG GCT TTC CAC TGC GAG GGG CTG TGC GAG TTC CCA TTG 1950
Leu Glu Tyr Glu Ala Phe His Cys Glu Gly Leu Cys Glu Phe Pro Leu
45 50 55

CGC TCC CAC CTG GAG CCC ACG AAT CAT GCA GTC ATC CAG ACC CTG ATG 1998
Arg Ser His Leu Glu Pro Thr Asn His Ala Val Ile Gln Thr Leu Met
60 65 70

AAC TCC ATG GAC CCC GAG TCC ACA CCA CCC ACC TGC TGT GTG CCC ACG 2046
Asn Ser Met Asp Pro Glu Ser Thr Pro Pro Thr Cys Cys Val Pro Thr
75 80 85

CGG CTG AGT CCC ATC AGC ATC CTC TTC ATT GAC TCT GCC AAC AAC GTG 2094
Arg Leu Ser Pro Ile Ser Ile Leu Phe Ile Asp Ser Ala Asn Asn Val
90 95 100 105

GTG TAT AAG CAG TAT GAG GAC ATG GTC GTG GAG TCG TGT GGC TGC AGG 2142
Val Tyr Lys Gln Tyr Glu Asp Met Val Val Glu Ser Cys Gly Cys Arg
110 115 120

TAGCAGCACT GGCCCTCTGT CTTCCTGGGT GGCACATCCC AAGAGCCCCT TCCTGCACTC 2202

CTGGAATCAC AGAGGGGTCA GGAAGCTGTG GCAGGAGCAT CTACACAGCT TGGGTGAAAG 2262

GGGATTCCAA TAAGCTTGCT CGCTCTCTGA GTGTGACTTG GGCTAAAGGC CCCCTTTTAT 2322

CCACAAGTTC CCCTGGCTGA GGATTGCTGC CCGTCTGCTG ATGTGACCAG TGGCAGGCAC 2382

AGGTCCAGGG AGACAGACTC TGAATGGGAC TGAGTCCCAG GAAACAGTGC TTTCCGATGA 2442

GACTCAGCCC ACCATTTCTC CTCACCTGGG CCTTCTCAGC CTCTGGACTC TCCTAAGCAC 2502

CTCTCAGGAG AGCCACAGGT GCCACTGCCT CCTCAAATCA CATTTGTGCC TGGTGACTTC 2562

CTGTCCCTGG GACAGTTGAG AAGCTGACTG GGCAAGAGTG GGAGAGAAGA GGAGAGGGCT 2622

TGGATAGAGT TGAGGAGTGT GAGGCTGTTA GACTGTTAGA TTTAAATGTA TATTGATGAG 2682

ATAAAAAGCA AAACTGTGCC T 2703

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 501 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Arg Leu Pro Lys Leu Leu Thr Phe Leu Leu Trp Tyr Leu Ala Trp
-380 -375 -370 -365

Leu Asp Leu Glu Phe Ile Cys Thr Val Leu Gly Ala Pro Asp Leu Gly
-360 -355 -350

Gln Arg Pro Gln Gly Thr Arg Pro Gly Leu Ala Lys Ala Glu Ala Lys
-345 -340 -335

Glu Arg Pro Pro Leu Ala Arg Asn Val Phe Arg Pro Gly Gly His Ser
-330 -325 -320

Tyr Gly Gly Gly Ala Thr Asn Ala Asn Ala Arg Ala Lys Gly Gly Thr
-315 -310 -305

Gly Gln Thr Gly Gly Leu Thr Gln Pro Lys Lys Asp Glu Pro Lys Lys
-300 -295 -290 -285

Leu Pro Pro Arg Pro Gly Gly Pro Glu Pro Lys Pro Gly His Pro Pro
-280 -275 -270

Gln Thr Arg Gln Ala Thr Ala Arg Thr Val Thr Pro Lys Gly Gln Leu
-265 -260 -255

Pro Gly Gly Lys Ala Pro Pro Lys Ala Gly Ser Val Pro Ser Ser Phe
-250 -245 -240

Leu Leu Lys Lys Ala Arg Glu Pro Gly Pro Pro Arg Glu Pro Lys Glu
-235 -230 -225

Pro Phe Arg Pro Pro Pro Ile Thr Pro His Glu Tyr Met Leu Ser Leu
-220 -215 -210 -205

Tyr Arg Thr Leu Ser Asp Ala Asp Arg Lys Gly Gly Asn Ser Ser Val
-200 -195 -190

Lys Leu Glu Ala Gly Leu Ala Asn Thr Ile Thr Ser Phe Ile Asp Lys
-185 -180 -175

Gly Gln Asp Asp Arg Gly Pro Val Val Arg Lys Gln Arg Tyr Val Phe
-170 -165 -160

Asp Ile Ser Ala Leu Glu Lys Asp Gly Leu Leu Gly Ala Glu Leu Arg
-155 -150 -145

Ile Leu Arg Lys Lys Pro Ser Asp Thr Ala Lys Pro Ala Ala Pro Gly
-140 -135 -130 -125

Gly Gly Arg Ala Ala Gln Leu Lys Leu Ser Ser Cys Pro Ser Gly Arg
-120 -115 -110

Gln Pro Ala Ser Leu Leu Asp Val Arg Ser Val Pro Gly Leu Asp Gly
-105 -100 -95

Ser Gly Trp Glu Val Phe Asp Ile Trp Lys Leu Phe Arg Asn Phe Lys
-90 -85 -80

Asn Ser Ala Gln Leu Cys Leu Glu Leu Glu Ala Trp Glu Arg Gly Arg
-75 -70 -65

Ala Val Asp Leu Arg Gly Leu Gly Phe Asp Arg Ala Ala Arg Gln Val
-60 -55 -50 -45

His Glu Lys Ala Leu Phe Leu Val Phe Gly Arg Thr Lys Lys Arg Asp
-40 -35 -30

Leu Phe Phe Asn Glu Ile Lys Ala Arg Ser Gly Gln Asp Asp Lys Thr
-25 -20 -15

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Val Tyr Glu Tyr Leu Phe Ser Gln Arg Arg Lys Arg Arg Ala Pro Leu
       -10                 -5                  1

Ala Thr Arg Gln Gly Lys Arg Pro Ser Lys Asn Leu Lys Ala Arg Cys
  5                  10                  15                  20

Ser Arg Lys Ala Leu His Val Asn Phe Lys Asp Met Gly Trp Asp Asp
                25                  30                  35

Trp Ile Ile Ala Pro Leu Glu Tyr Glu Ala Phe His Cys Glu Gly Leu
            40                  45                  50

Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn His Ala Val
        55                  60                  65

Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr Pro Pro Thr
    70                  75                  80

Cys Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu Phe Ile Asp
 85                  90                  95                  100

Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met Val Val Glu
                105                 110                 115

Ser Cys Gly Cys Arg
            120
```

国際様式 INTERNATIONAL FORM

〔特許手続上の微生物の寄託の国際的承認に関するブダペスト条約〕

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

下記国際寄託当局によって規則7．1に従い発行される

原寄託についての受託証

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page.

寄託者 氏名（名称） ヘキストジャパン（株）医薬研究開発本部 取締役本部長 丸山 博巳 殿

あて名 〒 350-11 埼玉県川越市南台1－3－2

I．微生物の表示

（寄託者が付した識別のための表示）	（受託番号）
MC-2	FERM BP- 5142

II．科学的性質及び分類学上の位置

I欄の微生物には、次の事項を記載した文書が添付されていた。

☒ 科学的性質

☒ 分類学上の位置

III．受領及び受託

本国際寄託当局は、平成 7年 6月 21日（原寄託日）に受領したI欄の微生物を受託する。

IV．移管請求の受領

本国際寄託当局は、 年 月 日（原寄託日）にI欄の微生物を受領した。
そして、 年 月 日に原寄託よりブダペスト条約に基づく寄託への移管請求を受領した。

V．国際寄託当局

通商産業省工業技術院生命工学工業技術研究所

名称： National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology

所長 大石道夫 Michio Oishi, D., DIRECTOR GENERAL.

あて名：日本国茨城県つくば市東1丁目1番3号（郵便番号305） 1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken 305, JAPAN

平成 7年（1995） 6月 21日

CLAIMS

1. A protein comprising amino acids 1 to 121 of SEQ ID NO.1 of the enclosed Sequence Listing.

2. Process for the production of a biologically active protein according to claim 1, wherein at least a part of a DNA-sequence as shown in SEQ ID NO:1 is introduced into a suitable host cell under conditions that allow expression of the DNA-sequence and protein formation, followed by isolation of said protein from other proteins produced by said host cell.

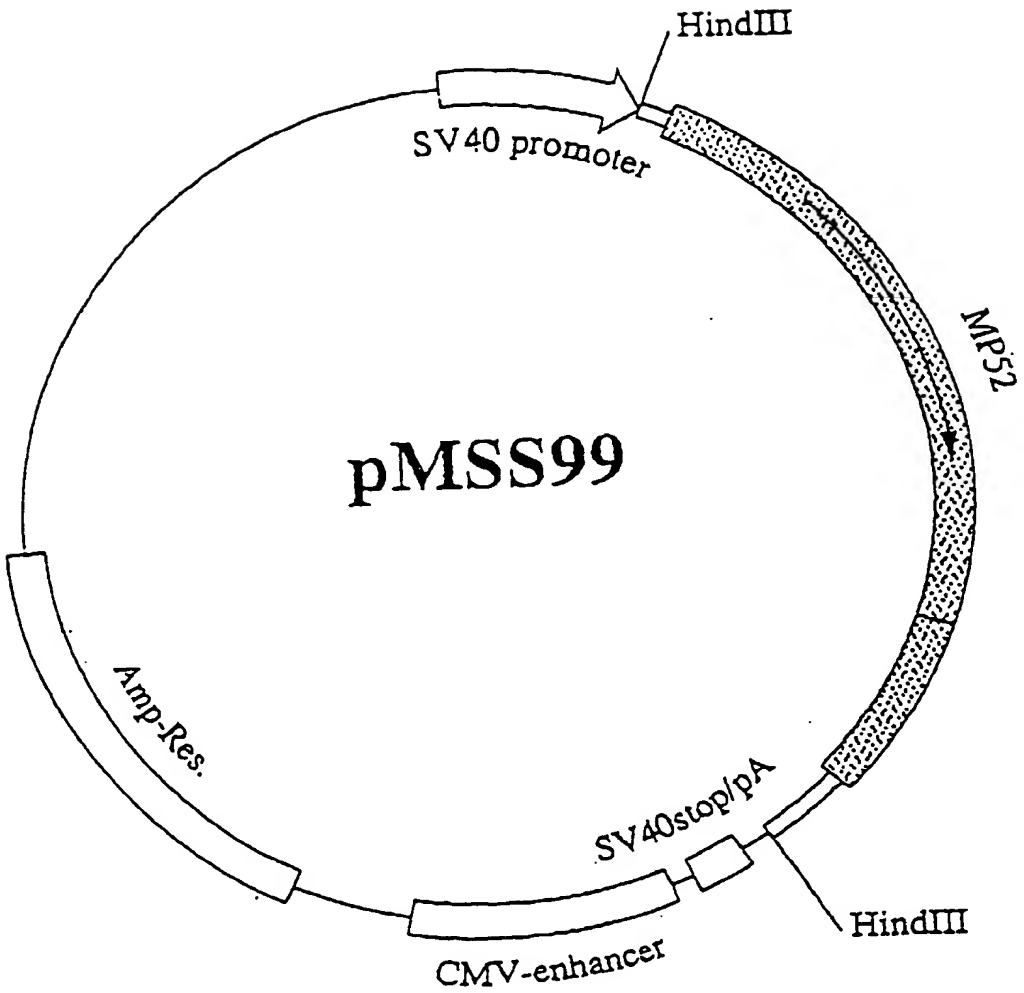
3. Pharmaceutical composition, containing as the active substance a protein according to claim 1, if desired together with usual carrier substances, auxiliary substances, diluents and fillers.

4. Use of a pharmaceutical composition according to claim 3 for promoting bone morphogenation, treatment or prevention of damage to bone, cartilage, connective tissues, skin, mucous membranes, epithelium or teeth, for application in dental implants and for application in wound-healing and tissue regeneration processes.

5. Use of a pharmaceutical composition as claimed in claim 3 for treating osteoporosis or bone fracture.

6. A parmaceutical composition as claimed in claim 3 for the use of orthopedic reconstruction, bone transplantation, cosmetic surgery or dental implantation.

Fig. 1


HindIII
SV40 promoter
MP52
pMSS99
Amp-Res.
SV40stop/pA
HindIII
CMV-enhancer

INTERNATIONAL SEARCH REPORT

al Application No
PCT/EP 96/03427

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/495 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE,A,44 20 157 (BIOPHARM GESELLSCHAFT ZUR BIOTECHNOLOGISCHEN ENTWICKLUNG VON PHARMAKA) 23 February 1995 see page 2, line 48 - page 3, line 1 see page 3, line 56 - page 4, line 36 & WO,A,95 04819 cited in the application ---	1-6
X	WO,A,93 16099 (BIOPHARM GESELLSCHAFT ZUR BIOTECHNOLOGISCHEN ENTWICKLUNG VON PHARMAKA) 19 August 1993 cited in the application see page 6, paragraph 2 - page 8, paragraph 1 see page 9, paragraph 2 ---	1-6
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 1997	22. 01. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,95 16035 (GENETICS INSTITUTE, INC.) 15 June 1995 see page 5, line 4 - line 6 see page 10, line 15 - page 11, line 3 see page 47; example 6 ---	1-6
X	NATURE, vol. 368, no. 6472, 14 April 1994, LONDON GB, pages 639-643, XP002018816 ELAINE E. STORM ET AL. : "Limb alterations in brachypodism mice due to mutations in a new member of the TGFbeta-superfamily" cited in the application see abstract; figure 1B see page 641, right-hand column, paragraph 2 - page 642, right-hand column, paragraph 1 ---	1-6
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 204, no. 2, 28 October 1994, ORLANDO, FL US, pages 646-652, XP002018817 GERTRUD HÖTTEN ET AL.: "Cloning and expression of recombinant human growth/differentiation factor 5" see abstract; figure 1 -----	1,2

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 96/ 03427

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 4,5
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 4 and 5, as far as concerning an in-vivo method, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invenuon first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/03427

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
DE-A-4420157	23-02-95	AU-A-	7498694	28-02-95
		CA-A-	2169171	16-02-95
		CZ-A-	9600357	17-07-96
		WO-A-	9504819	16-02-95
		EP-A-	0713529	29-05-96
		CN-A-	1129013	14-08-96
		ZA-A-	9405992	14-03-95
WO-A-9316099	19-08-93	AU-A-	3497193	03-09-93
		CA-A-	2129820	19-08-93
		CZ-A-	9401942	18-10-95
		EP-A-	0625989	30-11-94
		HU-A-	67683	28-04-95
		JP-T-	7503847	27-04-95
		NZ-A-	249113	26-07-96
WO-A-9516035	15-06-95	AU-A-	1301395	27-06-95
		CA-A-	2176942	15-06-95
		EP-A-	0733109	25-09-96
		FI-A-	962350	16-07-96
		NO-A-	962304	04-06-96